



Laboratory methods for diagnosing cryptosporidiosis

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Introduction

The laboratory diagnosis of cryptosporidiosis in man, for both clinical and epidemiological purposes, requires a knowledge of its natural history and pathogenesis. The biology of the organism and the clinical and epidemiological features of the infection have recently been reviewed in detail.¹⁻³

CLINICAL FEATURES

Cryptosporidium is now widely recognised as a cause of acute gastroenteritis, particularly in children. The infection in infants or the elderly is not particularly common, nor more severe. The infection usually produces a persistent, watery, offensive diarrhoea, often accompanied by abdominal pain, nausea, vomiting (especially in children), and anorexia; about a third of cases have other symptoms including clinically important weight loss, fever, and cough. Cryptosporidium is also a cause of severe and potentially life-threatening disease in the immunocompromised, especially those with AIDS. In these patients the infection sometimes affects the biliary tract, pancreas, or the respiratory tract. Oocyst excretion and symptoms, including the degree of diarrhoea, can fluctuate during the course of the disease. Many patients stop excreting oocysts within one to three weeks of the disappearance of symptoms, but some excrete for several weeks, and a few continue to excrete for months. Asymptomatic infection may be more commonly found in underdeveloped areas with poor hygiene, or where there is close and frequent contact with livestock.²

EPIDEMIOLOGY

Infection in man is derived, either directly or indirectly, from animals or from other infected people. Many cases are sporadic, but small clusters and family and community-wide outbreaks do occur. Seasonal peaks have also been noted in some areas. At times, and in some localities generally, the prevalence may be very low and hence the rate of detection in

laboratory specimens will also be very low (a fraction of 1%).² In such circumstances it may be considered unrewarding to look for the parasite except in selected cases.

The distribution of positive findings by age varies: positivity rates, by age range, of more than 62 000 patients studied in a two year Public Health Laboratory Service surveillance study are shown in the table.⁴

BIOLOGY OF CRYPTOSPORIDIUM

Infection in man and livestock is usually with *C parvum*. The biology and life cycle have been described in detail elsewhere.^{2,3} The life cycle is complex, with several characteristic stages of development after ingestion of an oocyst. It is important to recognise that only the oocyst stage is normally detected in stool specimens, although other stages can be found in other types of specimen such as biopsy tissues, and in sputum where they may be present in exfoliated cells (Casemore DP, unpublished observation). The oocyst is a well defined structure in which the component parts and ultrastructures, mainly typical of apicomplexan protozoa, can be shown by appropriate methods. The oocyst contains four naked spindle-shaped sporozoites which are released through a suture during excystation. Most of the features cannot, however, be visualised in any detail by conventional light microscopy. Differences in the degree of sporulation and the routes of sporogenous development may be responsible for some of the variation in staining characteristics and internal "structures" seen by light microscopy in stained preparations.

Laboratory diagnosis

Diagnosis is generally by means of detection of oocysts in faeces, and occasionally in other specimens; methods are described in detail below. The endogenous (tissue) stages can be shown by light and electron microscopical techniques in biopsy and necropsy tissues.

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Distribution of cryptosporidium positive stools by age*

Age group	n =	Crypto-sporidium positive	Percentage positive by age group	Percentage positive of all cryptosporidium positive stools	Accumulative rate per cent of all cryptosporidium positive stools
< 1	4090	79	1.9	6.1	6.1
1-4	9880	480	4.9	37.0	43.2
5-14	4822	210	4.4	16.2	59.4
15-24	8235	160	1.9	12.4	71.7
25-34	9048	155	1.7	12.0	83.7
35-44	6710	83	1.2	6.4	90.1
45-54	4992	32	0.7	2.5	92.6
55-64	3846	12	0.7	0.9	93.5
> 65	5432	21	0.4	1.6	95.1
NK	5866	63	1.1	4.9	—

*Data derived from Palmer and Biffin.²

STOOL EXAMINATION

Various methods have been described⁵⁻¹¹ for the detection and identification of oocysts in clinical specimens. Those described in detail here are commonly used and are generally reliable, given normal good laboratory practice, including the use of known positive controls. Measurement of putative oocysts is essential and a method is described below.

STAINING METHODS

Modified Ziehl-Neelsen staining was introduced for staining cryptosporidial oocysts by veterinary workers who had found that *Cryptosporidium* was associated with scouring of calves. They also recognised the problems associated with the Romanowsky staining methods.⁵ Phenol-auramine staining is very reliable, either when used as for staining mycobacteria or in a method developed for staining oocysts which is also widely used.⁸ A method using safranin and methylene blue has been described by Baxby *et al.*⁹ Care needs to be used in interpretation, particularly of preparations stained with modified Ziehl-Neelsen, as a variety of structures can be confused with oocysts (so-called cryptosporidium-like bodies). These include fungal spores which are generally larger (6-10 μ m) than oocysts (4-6 μ m), including mould spores (such as some *Mucor* species), and the spores of the common mushroom: fat globules and bacterial spores have also been mistaken for oocysts by some workers, although these can be distinguished clearly by size alone. Excessive Ziehl-Neelsen staining can result in false positive reactions from yeast cells.

IMMUNOLOGICALLY BASED METHODS

Detection and identification of oocysts can be achieved using monoclonal antibody immunofluorescence (IFAT):^{10,11} this often shows the characteristic suture line on the surface of the oocyst. Although the use of IFAT has been described for routine stool examination, this is not widely practised, partly because it is expensive. Two monoclonal antibodies are currently available commercially in the United Kingdom in kit form, both containing monoclonal antibodies specific for oocyst wall epitopes. A direct IFAT, developed in the United Kingdom,¹¹ is available from Northumbria Biologicals Ltd, Co Durham, and an indirect IFAT¹⁰ is available from Meridian Diagnos-

tics Inc, USA, and from Launch Diagnostics, Longfield, Kent. A conjugated monoclonal reagent is also available from the Public Health Laboratory Service from the Division of Microbiological Reagents.¹¹ None of these monoclonal antibodies is completely specific for *C. parvum*. Details of methods are provided in the manufacturer's instructions.

Other immunologically based antigen detection methods such as ELISA and passive agglutination, such as latex particle tests, have been described but have not, so far, proved sufficiently reliable and are not generally available.² Commercial ELISA tests are currently being evaluated and they may eventually replace microscopical methods in many laboratories.

Several authors have reported antibody detection methods which show that an immune response occurs with production of all of the main antibody classes.^{1,2,12} Such studies are useful epidemiologically and for the study of pathogenesis but are of little value for diagnosis in individual cases and are not described here.

Definitive identification of objects as oocysts can be made using transmission electron microscopy of thin sections of pelleted, embedded faecal samples,⁸ but this is of little practical value for diagnostic purposes.

CONCENTRATION METHODS

Faeces from patients with acute cryptosporidiosis do not usually require concentration to detect oocysts, although the numbers of oocysts excreted can fluctuate during the course of the infection. Examination of concentrated specimens may be merited in family contacts of index cases, or when this is required epidemiologically. Concentration of specimens may sometimes be indicated in the management of immunocompromised patients with a previous history of unexplained or uninvestigated diarrhoea. Such patients can occasionally experience remission and subsequent recrudescence of cryptosporidiosis.¹ Concentration methods described include sucrose flotation^{13,14} and formol-ether extraction.⁸ Use of an unmodified formol-ether method can result in loss of oocyst⁸: the modification described below was developed for recovery of cryptosporidial oocysts, but the concentrate can also be used for the detection of other parasites. Objects superficially resembling oocysts, except that they are osmotically less robust, have been reported to occur in some sucrose flotation preparations.¹⁵ Although associated with gastrointestinal symptoms, their nature and clinical importance has yet to be resolved (Baxby D, personal communication).

CONTROL OF INFECTION

It is not currently possible reliably to confirm clearance of the infection given the relative insensitivity of microscopical methods of detection compared with bacteriological enrichment culture. For those with confirmed cryptosporidiosis, enteric precautions are

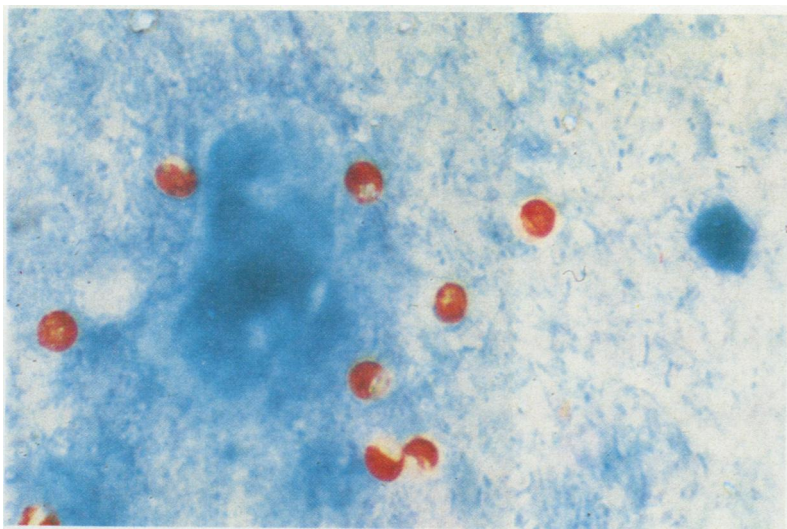


Figure 1 Faecal smear stained by modified Ziehl-Neelsen showing well stained oocysts, 4.5–5.0 µm.

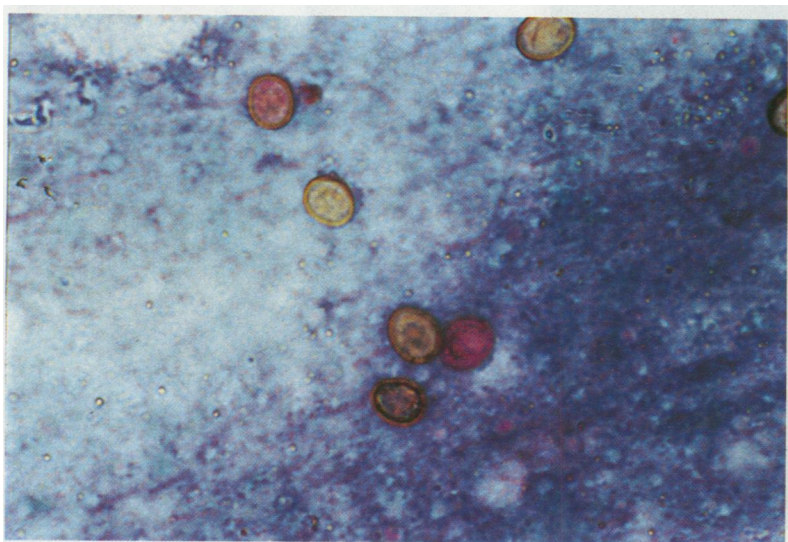


Figure 2 Faecal smear stained by modified Ziehl-Neelsen showing oocysts, 4.5–5.0 µm, with variable staining.

Figure 3 Faecal smear stained by modified Ziehl-Neelsen showing mushroom spores, about 6 × 8 µm.

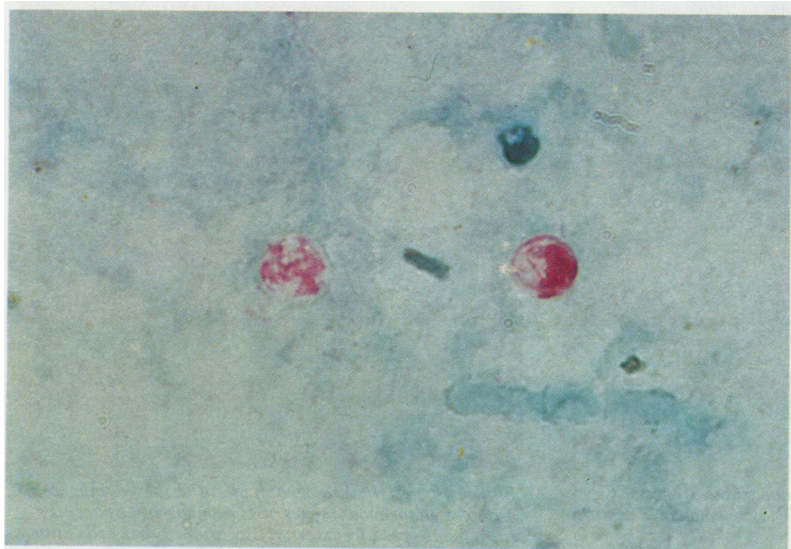


Figure 4 Faecal smear stained by modified Ziehl-Neelsen showing mould spores, about 8–10 µm.

advised while diarrhoea persists. Leave of absence from work for clinically recovered subjects is not required, provided that good standards of personal hygiene are maintained.¹⁶

Laboratory procedures

SAFETY

Clinical specimens should be handled with the care normally used for handling infectious material. It should also be recognised that cryptosporidiosis is common in patients with AIDS whose specimens may contain a variety of other infectious agents, including human immunodeficiency virus. Smears from suspected or confirmed AIDS patients can be immersed for one hour in freshly activated glutaraldehyde solution before staining. Oocysts are unusually resistant to most disinfectants but are killed by 10 vol. hydrogen peroxide.²³ They are very sensitive to the effects of heat, freezing, and desiccation.

Staining reagents can be hazardous to health and should be assessed and handled according to the CQSHH regulations and guidelines.

QUALITY ASSURANCE/CONTROL

A confirmed positive specimen should always be used to control staining and for comparative purposes. Stains need to be batch tested when a new batch is purchased and staining times may sometimes need to be adjusted accordingly. Doubtful or equivocal positive samples should always be checked by an alternative method and can be sent to a reference laboratory.

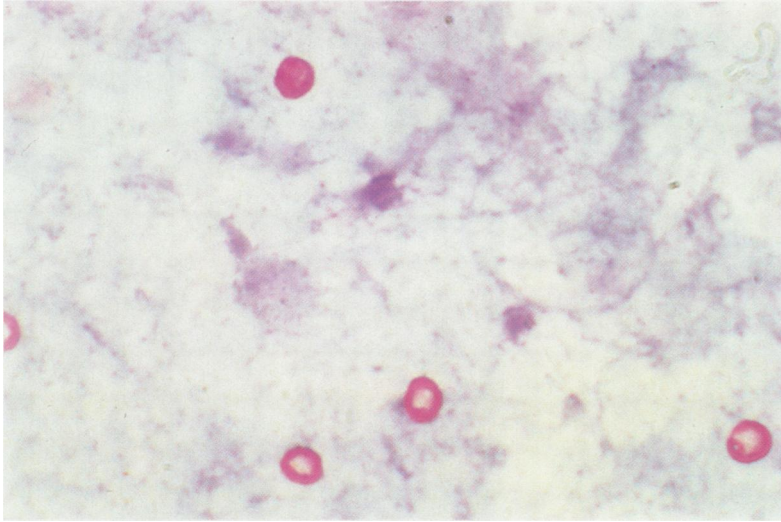


Figure 5 Sputum smear stained by modified Ziehl-Neelsen showing oocysts, 4.5–5.0 μ m, with 'erythrocyte' staining.

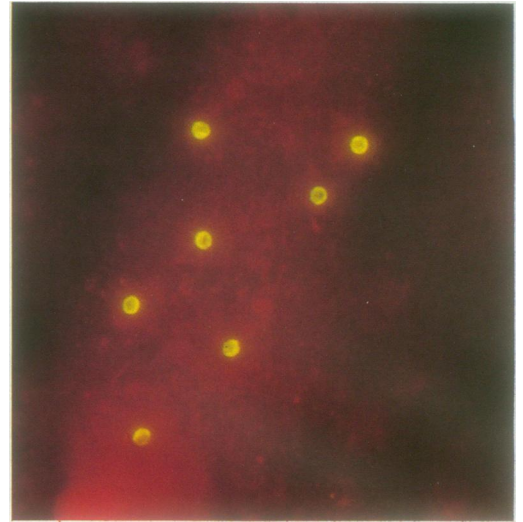


Figure 6 Faecal smear stained by auramine/carbol-fuchsin showing a group of oocysts with characteristic 'erythrocyte' pattern of staining.

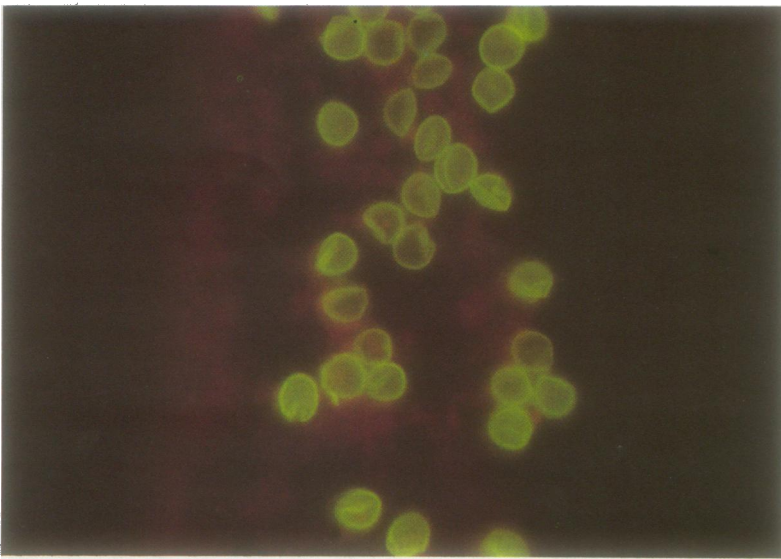


Figure 7 Purified oocysts of *C. parvum* stained by monoclonal antibody/FITC conjugate, counterstained with 0.1% Evan's blue, showing the suture on the oocyst surface.



Figure 8 Transmission electron-micrograph of an oocyst in a faecal pellet showing four sporozoites and a cytoplasmic residual body.

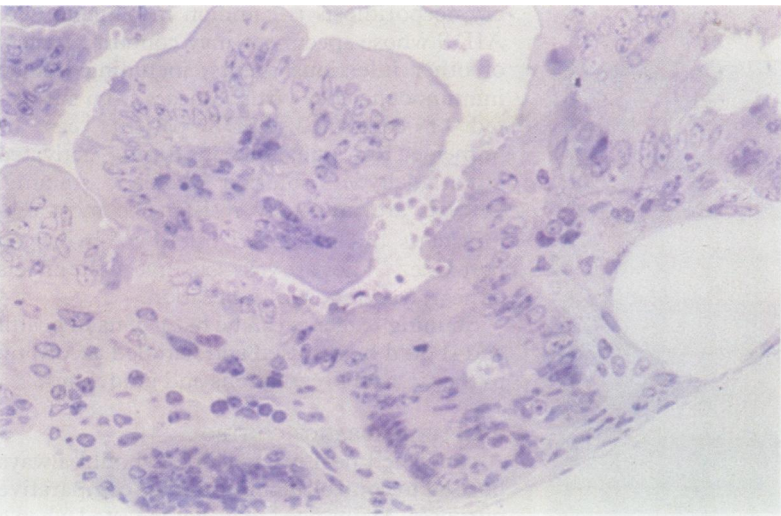


Figure 9 Light micrograph of gut tissue showing a group of endogenous stages of *C. parvum* in apparently superficial (pseudo-external) location. Plastic embedded semithin section stained by toluidine blue (Courtesy of Dr A Curry).

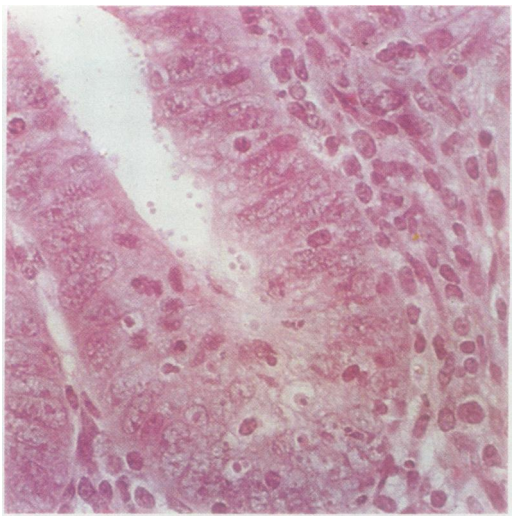


Figure 10 Light micrograph of gut tissue showing a group of endogenous stages of *C. parvum* in apparently superficial (pseudo-external) location. Some cells have apoptotic bodies (haematoxylin and eosin).

Staining in troughs, as described below, has not caused problems during more than seven years of experience in this laboratory. Positive results should be checked by examining a fresh smear stained by an alternative method before reporting.

SELECTION CRITERIA FOR SPECIMEN EXAMINATION

Ideally, all diagnostic specimens from patients with a history of acute or persistent gastroenteritis should be screened. Otherwise, selection must be based on clinical and epidemiological criteria to yield the greatest number of positive results. There is no validity in basing selection on the consistency of stool specimens submitted to the laboratory. Examination of the data on positivity by age shows that about 60% of positives will be found by screening specimens from children, and more than 90% by extending screening to include adults up to the age of 45 years (table). For epidemiological purposes, including outbreak control, data from adults can give an early indication of waterborne outbreaks.

DIRECT EXAMINATION OF FAECES

Macroscopic appearance should be noted but has little predictive value. Wet film examination of faeces from patients with acute cryptosporidiosis does not usually show the presence of pus cells or red blood cells unless there is concomitant infection with other organisms such as campylobacter; mucus is present in some. Oocysts cannot readily be identified in simple wet preparations, even in heavily infected specimens, and are difficult to distinguish reliably from yeast cells or fungal spores, particularly when the oocysts are present in small numbers. With experience such bodies can be distinguished in iodine wet-mounts, the oocysts remaining unstained, the yeasts staining brown. In Sheather's sucrose solution, with some optical systems, oocysts can have a pinkish hue compared with yeasts which appear greenish and in a lower focal plane. Specialised optical systems such as Nomarski Differential Interference Contrast (DIC) can also show internal structural details. In general, however, staining methods with fixed smears are to be preferred.

Staining methods

SLIDE PREPARATION

Smears, about 3 cm × 1 cm, are made on standard 3" × 1" glass microscope slides. The best results are obtained with moderately thick faecal smears made with the aid of a wooden applicator stick to give both thick and thin areas. The smears are allowed to air dry and can be lightly heat-fixed. Oocysts may be attached to mucus which tends to wash off the slide. It may help, with these and with non-fluid specimens, to emulsify a portion of the faeces in a little formol-water containing 0.1% Tween 80 (Casemore DP, unpublished observation).

1 Modified Ziehl-Neelsen (MZN)

Reagents The reagents are unmodified from those recommended for the Ziehl-Neelsen

staining of mycobacteria, as described in standard bacteriological texts. Kinyoun's modification can also be used.

Method The following modification has been found to be convenient and to give good results⁸: (i) slides are placed in multislide carriers for fixation and staining in batches in troughs. Fix in methanol for three minutes. (ii) Stain with strong carbol fuchsin for about 20 minutes (avoid excessive staining), followed by thorough rinsing in tap water. (iii) Give minimal decolourisation by agitation in a trough of 1% hydrochloric acid in methanol (15–30 seconds), followed by rinsing in tap water. (iv) Counterstain for 30–60 seconds in 0.4% malachite green (or methylene blue), rinse well, and air dry. (v) Examine by brightfield microscopy using × 50 and × 100 oil-immersion objective lenses. Some workers prefer to mount a coverslip on the smear (using immersion oil, or DPX mountant, or similar) and scan using a lower power dry objective lens. Suspicious bodies can then be further examined using an oil-immersion high power objective lens.

Results Oocysts are characteristically round or slightly ovoid with a consistent modal size, usually of about 4.5 × 5.0 µm (range 4–6 µm). They are acid-fast but oocyst staining, within a smear and between specimens, is very variable, and oocysts vary from unstained to partial red staining and complete staining: "erythrocyte" stained forms are common, and fully sporulated forms can be found in which red staining crescentic bodies, the sporozoites, can be seen within an unstained oocyst wall. (Note: *Cryptosporidium* in histological sections and empty, excysted, oocyst cases are not acid-fast).

2 Phenol-auramine/carbol-fuchsin method⁸

This simple two step method combines fluorescent staining using auramine with negative staining by strong carbol-fuchsin to mask background material. Handling time is brief and slides can be stained in multi-carriers as described above. Some workers use phenol-auramine staining, as recommended for mycobacteria or incorporate a brief acid-alcohol decolourisation step into the method described here.

Reagents Suitable reagents are those described in standard texts for the staining of mycobacteria: commercially produced solutions are generally satisfactory, but see note on quality control. The phenol-auramine formulation used in the author's laboratory is commercially prepared Lempert's solution and the carbol-fuchsin is that used for Ziehl-Neelsen staining, also commercially prepared.

Method (i) Slides are placed in multislide carriers for staining in batches in troughs, without fixation or decolourisation. Stain with phenol-auramine for 10–15 minutes, rinse well in tap water. (ii) Counterstain by immersing in strong carbol-fuchsin briefly (a few seconds), rinse well, and air dry. (iii) Examine by incident light fluorescence microscopy, using appropriate filters, with low and high power dry lens objectives.

Results Oocysts appear as bright yellow discs, often with an "erythrocyte" pattern of staining against a dark red background. Yeasts, fungal

spores, and other cryptosporidium-like bodies cannot readily be confused with oocysts in this method once the characteristic appearance of oocysts has been learned.

NB: Presumptive positive smears stained with phenol auramine (standard method or phenol auramine/carbol-fuchsin) can be confirmed, or equivocal fluorescent bodies examined further, by location using a Vernier reading or an England Finder slide, overstaining with MZN, and re-examining. Oocyst measurements should be made in this way—not on fluorescent objects.

Measurement of oocysts

Measurements are usually made with an eye-piece graticule calibrated by means of a micrometer scale on a microscope slide (Graticules Ltd, Tonbridge, Kent). The calibration procedure needs to be carried out only once for a given ocular and objective lens combination on a particular microscope. It is essential, however, to appreciate that the calibration is for that microscope and lens combination only and cannot be used for other lens combinations or with corresponding lenses on other microscopes, which must be separately calibrated.

Procedure (i) Place the eye-piece graticule on the focal plane diaphragm (between the eye lens and field lens) within the ocular lens. This graticule scale, usually 1 cm divided into 1 mm divisions, is an arbitrary transfer scale. Return the ocular lens to the microscope and focus the graticule using the eye-piece focusing ring, if present. (ii) Focus on the scale on a slide micrometer—usually 1 mm divided into 0.1 and 0.01 mm—using a low power objective lens to locate the scale. Change to the objective lens to be calibrated and adjust the position of the stage so that the “0” line of the two scales are superimposed and clearly focused. (iii) Taking care not to move the stage, look for other lines which are superimposed, as far along the scale as possible. Record the number of ocular divisions and the distance in mm on the slide between the superimposed sets of lines. (iv) Calculate the distance covered by the ocular graticule divisions as follows:

1 ocular unit in μm =

$$\left[\frac{\text{distance in mm covered}}{\text{number of ocular divisions}} \right] \times 1000$$

Thus for example, with a particular objective lens, if 0.25 mm on the micrometer slide scale is covered by 85 ocular divisions, one ocular division is equivalent to $2.47 \mu\text{m}$: an object covered by two divisions when viewed with that objective will thus be $4.84 \mu\text{m}$. (v) Repeat the process for each objective to be calibrated and record. The eye-piece graticule can conveniently be left in place.

Concentration

3 Modified formol-ether method⁸

Reagents (i) 10% formalin in water (formol-water), (ii) ether (note general comment on safety, above).

Method (i) Emulsify about 0.5 cm³ of stool in a

glass universal in a small volume of formol-water, using an applicator stick to break up formed stools if required, make up to about 3 cm³, and mix well using a vortex mixer. (ii) Add about 3 cm³ of ether and shake vigorously for 30–40 seconds. (iii) Make up to about 15 cm³ with formol-water, remix, and pour through a 40 mesh gauze sieve (Endecott's Ltd, London: certified test sieve, 3 inch ASTM, 425 μm) into a 15 ml glass conical centrifuge tube. (iv) Top up tube with formol-water if required, centrifuge in a safety bucket by setting centrifuge at $450 \times g$ (about 1000 rpm) and spin for one minute. (v) Using a disposable plastic bulb pipette, carefully remove column of fluid from between the ether layer and the deposit to a second tube. Top up the second tube with formol-water, centrifuge at $1000 \times g$ for 10 minutes, discard supernatant fluid and use deposit to prepare smears for staining in the usual way (the primary and secondary deposits can also be examined for ova and cysts of other parasites if required).

Histological diagnosis

Although the diagnosis of cryptosporidiosis is usually made microbiologically, identification of the endogenous (tissue) stages of the parasite in biopsy or necropsy tissues is sometimes required. The parasite can be seen in such material when processed and stained by conventional histological methods, such as haematoxylin and eosin staining of paraffin wax embedded tissue, although it can be inconspicuous and readily overlooked. Better results can be obtained by the method described in outline below (Curry A, personal communication).

Method (i) Fix about 1 mm³ pieces of tissue in 3% 0.1 M cacodylate buffered glutaraldehyde. (ii) Wash in buffer and then postfix in buffered (pH 7.2) 1% osmium tetroxide. (iii) Dehydrate in graded alcohols and embed in plastic resin and cut semithin (about 1 μm) sections. (iv) Stain with 1% aqueous toluidine blue, mount preparation, and examine by light microscopy. **Results** The parasite can be seen as small (2–8 μm), single-celled, round bodies in an apparently superficial, pseudo-external, location along the brush border, particularly on apical enterocytes and in the crypts. There may be some change in villous architecture and evidence of inflammation, and some cellular infiltrate into the mucosa.

Blocks thought to merit further detailed study and to confirm the identity of possible positives can be examined by transmission electron microscopy of ultrathin sections following staining—for example, by uranyl acetate and lead citrate. The various stages of the parasite have been described and elegantly illustrated by Current *et al.*^{3,17}

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